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Improved Diagnostic Medium for Separation of *Cryptococcus neoformans* var. *neoformans* (Serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (Serotypes B and C)

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A simple new agar medium containing L-canavanine, glycine, and bromthymol blue was found to give a clearer and more accurate distinction between serotype A or D (*Cryptococcus neoformans* var. *neoformans*) and serotype B or C (*C. neoformans* var. *gattii*) than creatinine-dextrose-bromthymol blue or glycine-cycloheximide-phenol red media. Among 143 isolates of serotype A or D and 70 isolates of serotype B or C, the new medium correlated completely with the serotype, whereas nearly 11% of these isolates gave discrepant reactions with creatinine-dextrose-bromthymol blue and glycine-cycloheximide-phenol red media.

A decision to divide *Cryptococcus neoformans* into two species, *C. neoformans* and *C. bacillisporus* (1), was reversed when a recent critical study on physiological, morphological, and pathological characteristics of *C. bacillisporus* (K. J. Kwon-Chung, J. E. Bennett, and J. C. Rhodes, Antonie van Leeuwenhoek J. Microbiol. Serol., in press) demonstrated the species to be synonymous with *C. neoformans* var. *gattii* (7). Kwon-Chung et al. (in press) then reclassified the species *C. neoformans* into two varieties: *C. neoformans* var. *neoformans* (serotypes A and D) and *C. neoformans* var. *gattii* (serotypes B and C). The perfect states of *C. neoformans* were also reduced into two varieties: *Filobasidiella neoformans* var. *neoformans* (*C. neoformans* var. *neoformans*) and *F. neoformans* var. *bacillispora* (*C. neoformans* var. *gattii*). Previous workers (2, 6) considered *C. bacillisporus* and *C. neoformans* to be conspecific and did not recognize the name *C. neoformans* var. *gattii*. Schmeding et al. (6) studied the sexual compatibility between *F. neoformans* and *F. bacillispora* and concluded that *F. bacillispora* is a synonym of *F. neoformans*. Unfortunately, these workers reported neither the viability of the progeny nor the evidence of meiosis obtained by such crosses.

A simple test for separating *C. neoformans* var. *neoformans* from the variety *gattii* was developed in 1978 (1). With creatinine-dextrose-bromthymol blue agar (CDB), only 4.4% of 90 *C. neoformans* var. *neoformans* isolates, but 100% of 46 *C. neoformans* var. *gattii* isolates, turned the medium blue by 48 h. The biochemical basis of the color change was studied by Polacheck and Kwon-Chung (4). The alkalinity which the pH indicator reflects was found to be a result of ammonia produced by creatinine degradation. Creatinine, in both varieties of *C. neoformans*, was degraded by an inducible enzyme, creatinine deiminase, into methylhydantoin and ammonia. However, in the isolates of *C. neoformans* var. *neoformans*, the presence of small amounts of ammonia repressed the synthesis of creatinine deiminase and prevented the further accumulation of ammonia.

Recently, Muchmore et al. (3) tested 108 isolates of *C. neoformans* obtained from Oklahoma. Of these, 12 produced blue color on CDB medium. We confirmed the blue or blue-green color reaction that these 12 isolates produced on CDB; but when these isolates were serotyped, 1 was serotype C, and the remaining 11 were serotype A. Since 1978, we have tested 104 more isolates obtained from various parts of the world. Among these, eight isolates of serotype B produced false-negative reactions on CDB agar. These eight isolates assimilated *l*-malate and grew poorly at 37°C as does the type isolate of *C. neoformans* var. *gattii* (Kwong-Chung et al., in press). With these observations, a need became obvious for a more effective medium to separate the two varieties.

Salkin and Hurd (5) developed a new color medium differentiating the two varieties. They used the combination of cycloheximide and glycine with phenol red as the pH indicator. They found that 88% of *C. neoformans* var. *gattii* isolates assimilated glycine as a sole source of carbon, whereas only 20% of the *C. neoformans* var. *neoformans* serotype A and none of sero-

TABLE 1. Color change of *C. neoformans* isolates on CGB, CDB, and GCP media

Isolate	No. of isolates tested	No. of isolates turning medium blue on:				No. of isolates turning medium red on:	
		CDB		CGB		GCP	
		2 days[a]	5 days	2 days	5 days	2 days	5 days
C. neoformans var. *neoformans*							
Serotype A	101	11 (11)	11 (11)	0[b]	0	0	0
Serotype D	37	3 (8)	3 (8)	0[c]	0	0[d]	0
Serotype AD	5	0	0	0	0	0	0
C. neoformans var. *gattii*							
Serotype B	50	42 (84)	42 (84)	46 (92)	50 (100)	43 (86)	45 (90)
Serotype C	20	19 (95)	19 (95)	17 (85)	20 (100)	16 (80)	17 (85)

[a] Results of the color change in media, disregarding that of colony, are read after 2- and 5-day incubation at 25°C. Numbers in parentheses are percentages.

[b] Two of 101 isolates produced a light green color around the culture when the inoculum was heavy.

[c] Two of 37 isolates produced a light green color around the culture when the inoculum was heavy.

[d] One of 37 isolates produced an orange-pink color.

type D isolates assimilated the amino acid. The isolates of *C. neoformans* var. *neoformans*, which assimilated glycine and would otherwise give a false-positive test, were sensitive to cycloheximide at the concentration of 1.6 μg/ml. All isolates of *C. neoformans* var. *gattii*, on the other hand, were resistant to the drug at the same concentration.

In our experience, cycloheximide susceptibility of the two varieties, measured by the agar dilution technique, was not significantly different. Of 29 isolates of *C. neoformans* var. *gattii*, 14% were sensitive to the drug at 1.6 μg/ml compared with 23% of 30 *C. neoformans* var. *neoformans* isolates. In comparative studies with 70 isolates of *C. neoformans* var. *gattii*, it was noticed that the glycine-cycloheximide-phenol red (GCP) medium of Salkin and Hurd gave less ambiguous results between positives and negatives than did CDB medium. However, the false-negative percentage with GCP was as high as with the CDB medium.

We report here a modification of CDB agar which effectively eliminated the false-positive and false-negative reactions by the two varieties. The glucose and creatinine in the CDB agar were replaced by 1% glycine as a sole source of carbon and nitrogen. The arginine analog, L-canavanine (30 μg/ml), was incorporated into the medium for reasons to be elucidated later in this report. This modified medium is designated as canavanine-glycine-bromthymol blue agar (CGB) and was prepared as follows. For the glycine–L-canavanine solution (solution A), 10 g of glycine, 1 g of KH_2PO_4, 1 g of $MgSO_4$, 1 drop (50 μl) of vitamin solution (Bejectal with vitamin C, Abbott Laboratories, North Chicago, Ill.), and 30 mg of L-canavanine sulfate (Sigma Chemical Co., St. Louis, Mo.) were added to 100 ml of distilled water. The Bejectal solution can be replaced by 1 mg of thiamine-HCl. The pH was adjusted to 5.6, and the solution was filter sterilized (0.45 μm of Nalgene). Aqueous bromthymol blue (0.4% [wt/vol]; solution B) was prepared either by dissolving 0.4 g of sodium bromthymol blue in 100 ml of distilled water or by dissolving 0.4 g of bromthymol blue in 64 ml of 0.01 N NaOH and adding 36 ml of distilled water. To prepare a liter of the medium, 880 ml of water, 20 ml of solution B, and 20 g of agar were mixed in a large flask and autoclaved for 15 min at 15 lb/in^2. While the agar solution was still molten, 100 ml of solution A was added and stirred thoroughly before pouring into petri dishes or dispensing aseptically into tubes to make slants. A positive test with CGB medium indicates a change in pH from 5.8 ± 0.1 (greenish yellow) to at least 7.0 (cobalt blue).

A total of 213 isolates of *C. neoformans*, including the two varieties, were tested on the CGB, CDB, and GCP agars. The results are shown in Table 1. The CGB agar was superior to the latter two media in that by day 5, 100% of *C. neoformans* var. *neoformans* could be differentiated from *C. neoformans* var. *gattii*. None of 143 *C. neoformans* var. *neoformans* tested produced blue color. Four of the 143 cultures produced a change in the agar around the culture from greenish yellow to light green within 48 h. A color change only occurred when the inoculum was heavy (a heaping loopful). These cultures stayed green, never turning blue, for at least 10 days. On CDB medium, 10% of *C. neoformans* var. *neoformans* produced blue or blue-green color by 48 h. On GCP agar, 1 of 143 *C. neoformans* var. *neoformans* produced an

orange-pink color by 48 h. These isolates would be confused with *C. neoformans* var. *gattii* if serotyping were not performed. Of 70 *C. neoformans* var. *gattii* isolates, 90% produced blue color on CGB agar by 48 h, whereas 87% of them did so on CDB agar. On GCP agar, 84% of these isolates produced red color by 48 h. After a 5-day incubation, all of the isolates of the variety *gattii* produced cobalt blue color on the entire surface of CGB agar compared with 87% on CDB agar. Eighty-nine percent of the isolates gave positive reactions on GCP medium by 5 days.

The eight isolates which gave false-negative results on GCP were found to be sensitive to cycloheximide at the concentration of 1.6 μg/ml. This may account for the failure to produce a color change. As previously reported, *C. neoformans* var. *gattii* isolates are all resistant to L-canavanine up to or exceeding 960 μg/ml, whereas all serotype D and 33% of serotype A isolates of *C. neoformans* var. *neoformans* are sensitive to the drug at 5 μg/ml or less (I. Polacheck and K. J. Kwon-Chung, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, F52, p. 328). The L-canavanine–resistant serotype A isolates did not assimilate glycine.

CGB is easy and inexpensive to prepare. Stock solutions can be filter sterilized and kept in the refrigerator for at least 6 months. The adjustment of pH is needed only for the stock solution A. Whether one uses bromthymol blue or sodium bromthymol blue as the indicator, the final pH comes out as 5.8 ± 0.1. The age and size of inoculum is not critical, and the culture can be incubated at any temperature between 25 and 30°C. However, to avoid green coloration by some isolates of *C. neoformans* var. *neoformans*, we recommend using less than a full loopful of cells for an inoculum. The CGB agar plates or slants can be stored in the refrigerator for at least 3 months without losing their efficacy. The GCP medium, however, appeared to be unstable in the refrigerator. When the GCP agar plates were tested after 3 months of storage in a refrigerator, three of five previously positive *C. neoformans* var. *gattii* isolates gave negative results. The lack of false-negative or false-positive results among 213 isolates indicated the superiority of CGB agar over any existing medium for the separation of the two varieties.

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